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ORIGINAL ARTICLE

Impact of male condition on his spermatophore and consequences for female reproductive performance in the Glanville fritillary butterfly

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Abstract In butterflies, male reproductive success is highly related to the quality and the size of the spermatophore transferred to the female. The spermatophore is a capsule produced by the male during copulation, which in many species contains sperm in addition to a nuptial gift, and which is digested by the female after copulation. The nuptial gift may contribute to egg production and offspring quality, and in some cases also to female body maintenance. The production of the spermatophore, however, represents a cost for the male and, in polyandrous species, ejaculates are sometimes allocated adaptively across matings. Nonetheless, although the ecological factors affecting the reproductive success of female butterflies have been the topic of numerous studies, little information exists on the factors affecting males' contribution to reproduction, and the indirect impacts on female fecundity and fitness. We used the Glanville fritillary butterfly, *Melitaea cinxia* (Linnaeus, 1758) (Nymphalidae), in order to assess variation in male allocation to matings. In this species, smaller males produce smaller spermatophores, but variation in spermatophore size is not correlated with female reproductive success. We show that spermatophore size increases with male age at first mating, decreases with mating frequency and adult food-deprivation, and is not influenced by developmental food-limitation. The length of copulation period does not influence the spermatophore size nor influences the polyandrous mating behavior in this species. Male contribution to his spermatophore size is clearly influenced by his condition and adult-resource at the time of mating. Despite this variation, spermatophore size does not seem to have a direct impact on female reproductive output or mating behavior.

Key words ejaculate; fecundity; food-restriction; *Melitaea cinxia*

Introduction

Many species have evolved flexible reproductive strategies allowing individuals to optimize their fitness by adjusting their reproductive effort in response to their state or environmental condition (Svensson & Sheldon, 1998;

Fox & Czesak, 2000). In general, female reproductive strategies, at least in species with no direct paternal care, have been of particular interest, as reproduction is often more costly in females than in males (Service, 1989; Gilg & Kruse, 2002; Kemp & Rutowski, 2004; Arnqvist & Rowe, 2005). Factors such as temperature and resource availability and/or quality can greatly influence the female reproductive investment in both egg size and egg numbers (Mangel, 1987; Thompson & Pellmyr, 1991; Ernsting & Isaaks, 1997; Fox & Czesak, 2000). Although reproductive investment decisions form an integral part of

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life-history biology in insects, we only start to understand the ecological factors that contribute to the plasticity of the male investment in reproduction. Male insects may contribute to female egg production and somatic maintenance by transferring a spermatophore, which contains sperm, hormones and male-derived nutrients, during copulation (in Lepidoptera: Boggs & Gilbert, 1979; Boggs, 1981; Andersson *et al.*, 2000; Bonoan *et al.*, 2015; in Coleoptera: South & Lewis, 2012; in Orthoptera: Voigt *et al.*, 2008). In various butterfly species a larger spermatophore is correlated with increased female fecundity (Rutowski *et al.*, 1987), lifespan (Simmons, 1990; Oberhauser, 1997; Pauku & Kotiaho, 2005), and offspring quality and survival (Wiklund *et al.*, 1993; Jones *et al.*, 2000; Karl & Fisher, 2013), and a larger spermatophore also correlates with a longer refractory period (Sugawara, 1979; Kaitala & Wiklund, 1995; Karl & Fisher, 2013). Therefore, any factor that might affect the size and/or quality of the transferred spermatophore may have indirect effects on both female and male reproductive performance.

In Lepidoptera, male spermatophores contain nonfertile (apyrene) sperm (up to 95% of the ejaculate, Cook & Wedell, 1996), fertile sperm (eupyrene), as well as a nutrient-rich nuptial gift (Meves, 1902). Similarly to the fact that egg production is costly for the female, the production of the spermatophore represents a cost for the male (Ferkau & Fischer, 2006). Typically in Lepidoptera, larger males produce larger spermatophores (Forsberg & Wiklund, 1989; Svård & Wiklund, 1989; Bissoondath & Wiklund, 1996), indicating biological restrictions in the production of large spermatophores. Furthermore, although an increase in the resting period between 2 consecutive matings often leads to an increase in the size of the subsequent spermatophore, the second spermatophore always remains smaller than the first one, as is the case in the Glanville fritillary butterfly, *Melitaea cinxia* (Duploux & Hanski, 2015). Additionally, the size of the spermatophore is known to decrease with male mating frequency. This has been shown in numbers of Lepidoptera including the Ant-tended lycaenid butterfly, *Jalmenus evagoras* (Hughes *et al.*, 2000), the Speckled wood butterfly, *Pararge aegeria* (Vande Velde *et al.*, 2011), and the oriental peach moth, *Grapholita molesta* (de Morais *et al.*, 2012). After several matings, males may also experience fatigue, which biological signs include either the need for a recovery period before producing another spermatophore (Kaitala & Wiklund, 1995; Bissoondath & Wiklund, 1996), an increased copulation length (Hughes *et al.*, 2000, but see Watanabe *et al.*, 1997 for contradicting results in the Sulfur butterfly), and sperm depletion or increased proportion of nonfertile sperm in the ejaculate

leading to lower paternity (Charlat *et al.*, 2007; de Morais *et al.*, 2012; Kehl *et al.*, 2015). However, as shown in the small heath butterfly, *Coenonympha pamphilus* (Cahenzli & Erhardt, 2013), males may also improve their reproductive output (offspring hatching mass) by feeding on nectar and transferring amino acid-rich spermatophores during reproductive period.

The Glanville fritillary butterfly has a wide geographic distribution in Europe (Nieminen *et al.*, 2004). Females are mostly monandrous, but polyandry has been reported in relatively low frequencies both in the wild (Boggs & Nieminen, 2004) and under seminatural conditions (Sarhan & Kokko, 2007; Duploux *et al.*, 2013). In this species, female reproductive effort has been well studied, with both life-history and genetic variation having a great impact on cumulative egg production, clutch size or egg hatching rate (Hanski & Saccheri, 2006; Saastamoinen, 2007a; Mattila *et al.*, 2012). In contrast, not much is known about the variation in male reproductive performance. We know that males are often polygynous, and the size of the spermatophore increases with pupal weight, with the first spermatophore being the largest (Duploux & Hanski, 2015). Here we further investigate paternal characteristics that may affect the male reproductive investment. We test if the size of the male spermatophore varies according to male age and food-restriction experienced at larval and adult stages, and consequently affect the female reproductive output (clutch size and egg hatching-rate). We show that male quality at the adult stage most impacts the size of the spermatophores produced during mating. However, the size of the spermatophore does not correlate with female reproductive output, copulation period or female remating behavior.

Materials and methods

Study species

The Glanville fritillary butterfly (*Melitaea cinxia* L. 1758, Lepidoptera: Nymphalidae) is found across Europe, North Africa and West Asia (Kuussaari *et al.*, 2004). In Finland, the species occurs on the Åland Islands ($N \sim 10\,000$), an archipelago between the coasts of Finland and Sweden in the Baltic Sea (Hanski *et al.*, 1995). Only a relatively small fraction of females (6%–8%) mate twice in the wild (Kuussaari, 1998) and under seminatural conditions (15%–22%, Sarhan & Kokko, 2007; Duploux *et al.*, 2013). Males, on the other hand, often mate more than once (36%, Wahlberg, 1995), and based on experiments conducted under seminatural field conditions males can mate more than 3 times and

still sire fertile eggs (Wahlberg, 1995; Duploux *et al.*, 2013). In all experiments, the larvae were maintained in diapause in incubators (12 h : 12 h day and night at 4 °C) until the following spring, and then reared to adult stage at the butterfly rearing facilities at the Lammi Biological Station (12 h day: 28 °C, 12 h night: 15 °C).

Set 1: Copulation length and female reproductive output

The adult butterflies released under seminatural field conditions in large outdoor cages were first collected as prediapause larvae from 211 family nests in 108 different habitat patches in 3 communes within the Åland Islands (Föglö, Saltvik, and Sottunga) during the fall 2012 (see details of habitat patches and communes in Ojanen *et al.*, 2013). Postdiapause larvae were reared in family groups in the laboratory and fed *ad libitum* on their natural host plant *Plantago lanceolata* until pupation. We recorded the weight of all individuals at pupal stage. Two days after eclosion, each butterfly was labeled with a unique number on the hind wing and released in 1 of 2 large outdoor cages (32 × 26 × 3 m each). The first cage received all individuals from Saltvik and Sottunga communes ($N_1 = 149$, including 70 females and 79 males from 107 families, with no more than 3 individuals from the same family), while the second cage received all individuals from Föglö ($N_2 = 221$, 120 females and 101 males from 104 families, with no more than 3 individuals from the same family).

The butterflies mated freely in the outdoor cages until death. In order to correlate spermatophore size with other life-history traits of the mating pairs (e.g., female and male fecundity traits), the cages were under constant survey during the butterflies' active daily period, from 8:00 am to 18:00 pm, to find all occurring matings. For each mating, we record the IDs of the males and females paired, as well as the time of the day. A small cage was then carefully placed on top of the butterfly pair and the butterflies were left undisturbed until the mating ended. The end time of the mating was recorded and the total length of the mating (copulation period) was calculated.

The large outdoor cages covered an artificial dry meadow closely resembling the natural habitat of the Glanville fritillary (Hanski *et al.*, 2006; Duploux *et al.*, 2013; Ojanen *et al.*, 2013), but from which all potential host plants had been removed prior to the experiment. Instead, females were provided with 200 potted host plants to oviposit on, 100 individuals of *P. lanceolata* and *V. spicata* placed in the central part of the cage. The plants were constantly monitored during the day in order to record the female ID, date and host plant of each oviposition. Each

clutch-carrying leaf was removed from the plant at the end of the oviposition, placed in a Petri dish and incubated in the laboratory. To minimize the risk of damaging the fragile eggs, the size of each clutch was measured by counting the number of eggs laid 3 d after oviposition, once the eggs are more robust. Similarly, the number of hatching caterpillars was counted 3 d after eclosion to minimize damage on the small caterpillars. Hatch rate was determined as the proportion between the number of larvae and number of eggs laid. The full bodies of 51 mated females (from 46 families, with only 5 families represented by a maximum of 2 females) were recovered from the outdoor cages at the end of the experiment (after 10 sunny days), and dissected as described below. The bodies of the other individuals were lost to predators (e.g., spiders and ants) present in the seminatural conditions of the outdoor cage.

Set 2: Larval and adult food-restriction and male age at mating

The individuals used in the 2 food-restriction experiments (either larval or adult food-restriction) were the F1 laboratory generation from wild larvae (F0) collected in 2013. In brief, fifth instar diapausing larvae (F0) were collected from winter nests in the communes of Saltvik, and reared *ad libitum* on *P. lanceolata* in the laboratory (12 h day: 28 °C, 12 h night: 15 °C) until adult stage. Randomly mated F0 females laid eggs on either host plant *P. lanceolata* or *Veronica spicata*. Hatching F1 larvae were reared in family groups *ad libitum* on *P. lanceolata* (12 h day: 28 °C, 12 h night: 15 °C) until diapause, and placed into incubating chambers for winter (day and night at 4 °C). In the spring 2014, 464 F1 larvae were awoken from diapause and reared in family groups *ad libitum* on *P. lanceolata* (12 h day: 28 °C, 12 h night: 15 °C) until they moulted for their final seventh instar. After measuring the weight of all seventh instar larvae, larvae were individually placed into a small container ($V = 100$ mL) and assigned to 1 of the 2 food-restriction experiments (see below). All individuals were again weighed 1 d after they pupated. After eclosion, no more than 20 adult butterflies were included in any of the mating cages (sex-ratio varying between 1 female for 2–3 males). We also avoided inbred matings by placing males and females of the same family into different cages. In the laboratory, matings are very much weather-prone, such that mature males and females mate in the indoor cages only on bright sunny days (Suvi Ikonen, pers. comm.). Consequently, each small mating cage included butterflies of mixed ages, with mated individuals being replaced with newly emerged virgin individuals

(See below for more details about each experiment). All individuals were frozen to death right after their first and only mating.

Set 2a: Larval food-restriction In order to test the effect of male food-restriction during final stages of development on adult reproductive performance larvae were split between control ($n = 115$) and food-restriction treatment ($n = 90$) on the day they moulted into the seventh instar. Larvae from the control group were fed *ad libitum* on their host plant *P. lanceolata*, whereas the larvae in the food-restriction treatment experienced 2 d of starvation with food available between the starvation days (i.e., larvae were starved on day 2 and 4 of their seventh instar), after which they were again fed *ad libitum* until pupation. Females emerging from the larval food-restriction group were discarded and not used in the mating experiment.

Fifty-three control females, from 9 families, were offered a mate, immediately after emergence, with control ($n = 62$) or larval food-restriction ($n = 53$) males from up to 10 families. At the time of the mating, the youngest females were 1 d old and the oldest were 6 d old (average: 1.7 d old), while the youngest males were 1 d old, and the oldest were 9 d old (averages: 4.9 and 4.6 d old for control and larval food-restriction males, respectively). This is within the age range reported from previous studies on the same species (under seminatural conditions the females are between 1.5 and 4 d old at the time of their first mating (Saastamoinen, 2007b; Duploux *et al.*, 2013), while males can mate on their date of emergence to at least 9 d old (Duploux *et al.*, 2013; Duploux & Hanski, 2015).

Set 2b: Adult food-restriction In order to test the effect of male food-restriction at adult stage, 259 larvae were fed *ad libitum* on their host plant *P. lanceolata* and weighed 1 d after pupation. Once eclosed, 53 males were food-restricted (supplied only with water) whereas the rest (controls; 64 males and 143 females) were fed *ad libitum* on a 1 : 5 honey and water solution. Females (all controls) from 10 families were given the opportunity to mate in small indoor mating cages containing either control or adult food-restricted males, from 10 families. Not all reared individuals mated, and at the time of mating, females were 1–3 d old (on average 1.4 d old), while males were 1–7 d old (on average 3.7 and 3.1 d old for control and food-restriction group, respectively).

Spermatophore size measurements

The spermatophore is produced by the male inside the female during copulation. At the end of the copulation, the female transfers the sperm and starts digesting the

spermatophore capsule, which remains in the bursa copulatrix and can be found by dissecting the female's abdomen. We dissected the abdomen of each female butterfly under a microscope (Nikon SMZ800, Tokyo, Japan), using sterile toothpicks, to isolate the spermatophore(s). The bursa copulatrix containing the spermatophore was dissected out of the female's abdomen, and was carefully opened to also record the chronological order of each spermatophore in case the female had mated several times. In contrast with the females from the larval and the adult food-restriction experiments, which only received 1 spermatophore, 11 females from the 2013 outdoor cage mated several times (7 females mated twice, 3 mated 3 times, and 1 female 4 times). Using a digital camera (MQA21010 with DS-L2 MQA110105.0MP, colour digital head DS-Fi1; Nikon), we separately photographed each spermatophore on top of a millimetre paper for scale. Using ImageJ (National Institutes for Health, Bethesda, MD, USA), we measured the length and width of the spermatophores from the food-restriction experiments (Set 2a and b) and calculated their surface area using the formula of an ellipse ($A = \pi ab$; with a = length of semimajor axis and b = length of semiminor axis) (Duploux & Hanski, 2015). For the copulation length and female reproductive output experiment (Set 1), the females laid several clutches before death, the dissected spermatophores were often partially digested and had lost their elliptic shape, therefore, we measured their cross section surface area, instead of measuring the length and width of the spermatophore. A subset of spermatophores from each experiment was measured twice to test for repeatability ($n = 42$, R^2 Set 1 = 0.96; $n = 30$, R^2 Set 2 = 0.97).

Sperm pictures

A spermatophore was placed in a drop of modified Barth saline solution (Gurdon, 1991) on a cavity slide, and carefully opened with a thin needle to gently stir out the content in the saline solution. We washed off the cavity slide into a 30 mL tube using a Barth saline solution and diluted with distilled water. We then gently shook the collecting tubes to ensure proper dispersal of the sperm cells. We placed a 10 μ L sample on a microscope slide and allowed it to dry. The dry slide was then dipped for 3 sec in distilled water to remove salt crystals and dried again under dust cover. We photographed the observed eupyrene and apyrene sperms using dark-field phase contrast microscopy (63 \times magnification).

Statistical analyses

Statistical analyses were carried out using R (R Core Team, 2013). In all of the analyses, we used backward

model selection by starting with a full model for each trait and sequentially eliminating interaction terms with the highest P value. Duploup and Hanski (2015) previously showed a correlation between spermatophore size with male pupal weight, while other studies (Saastamoinen, 2007a, 2008) showed that oviposition traits, such as clutch and egg numbers, are correlated to female pupal weight in the Glanville fritillary butterfly. When relevant, in the models described below, we used the spermatophore surface area and the clutch/egg number corrected for male or female pupal weight, respectively.

Set 1: Copulation length and female reproductive output We first investigated the effects of different male and female characteristics on spermatophore size. We used a linear mixed model to test the effect of male mating status on the size of the spermatophore (corrected for male pupal weight and male age at mating), with the respective female mating status as additional covariates, and male ID and cage (1 or 2) as random factors to the model. We tested whether the length of the mating period was influenced by male mating status (first, second, or third mating) or male age using 2 independent Kruskal–Wallis tests. Then, we used a linear mixed model to test the effect of the copulation period on the size of the first spermatophore only (corrected for male pupal weight and male age at mating), with cage (1 or 2) as a random factor to the model.

Second, we investigated whether spermatophore size or copulation period may influence remating behavior of the 11 females that mated once or more in the outdoor cage. We used a linear model to test the correlation between the size of the first spermatophore (mm^2) and female behavior (monandrous vs. polyandrous). Then, we used a Kruskal–Wallis test to investigate the effect of length of the first mating on female remating behavior.

Finally, we investigated which aspects of the matings may affect two female reproductive traits, including lifetime number of clutches and egg hatch rate. We used a linear model to test the effect of spermatophore surface area (mm^2) on the number of clutches a female laid (corrected for female pupal weight), only including females that had only mated once in the cage. Then, using linear mixed models, we tested the effect of spermatophore area (mm^2) on the average egg hatch rate of each female, and on the hatch rate of the first clutch laid after a new mating. In both cases, mating status of both male and female was included as covariates, and female family as a random factor. For both models, the egg hatch rate data was arcsin-corrected prior to analysis.

Set 2: Larval and adult food-restriction and male age at mating We used linear mixed models to examine first the effect of larval food-restriction on male pupal weight, with male family as a random factor, and second the effect of male larval and adult food-restriction on the size of the spermatophores (corrected for male pupal weight and age at mating), with experimental stage (adult or larvae) and male food treatment (control or restriction) included as covariates, and male family included as a random factor in the model. A *post hoc* test (Tukey's Honest Significant Difference test) was used to compare the effect within the interaction between experimental stage and treatment.

Data accessibility

Data files will be available in *Dryad* under the data package: <http://dx.doi.org/10.5061/dryad.2808g>.

Results

Spermatophore size

We measured a total of 193 spermatophores across all experiments. The 51 mated-females recovered from the copulation experiment in outdoor-cages (Set 1: 12 from cage 1 and 39 from cage 2) provided a total of 65 spermatophores, as 9 females had mated more than once. The other females were either unmated, or their bodies were not recovered from the outdoor cages. From the larval food-restriction experiment, 47 females mated, including 24 crosses with control males and 23 crosses with larval food-restricted males. From the adult food-restriction experiment, 85 females mated, including 43 crosses with control males and 42 crosses with food-restricted males. Typically, larger males produced larger spermatophores (linear mixed model, $F_{1,115} = 13.08$, $P < 0.001$ for first spermatophores only, and linear mixed model, $F_{1,101} = 6.01$, $P = 0.016$ for all spermatophores, Fig. 1A), and older males, at the time of the mating, also produced larger spermatophores (linear mixed model, $F_{1,101} = 9.92$, $P = 2.2\text{e-}3$ for first spermatophores only, and linear mixed model, $F_{1,115} = 9.5$, $P = 2.6\text{e-}3$ for all spermatophores, Fig. 1B). Average data can be found in Table 1.

Set 1: Copulation length and female reproductive output The size of the spermatophore decreased with male mating status (linear mixed model, $F_{1,9} = 9.39$, $P = 0.014$, Fig. 2A). The first mating was on average shorter than the consecutive matings (Kruskal–Wallis test, $\chi^2 = 6.62$, $\text{df} = 2$, $P = 0.036$, Fig. 2B), however, the

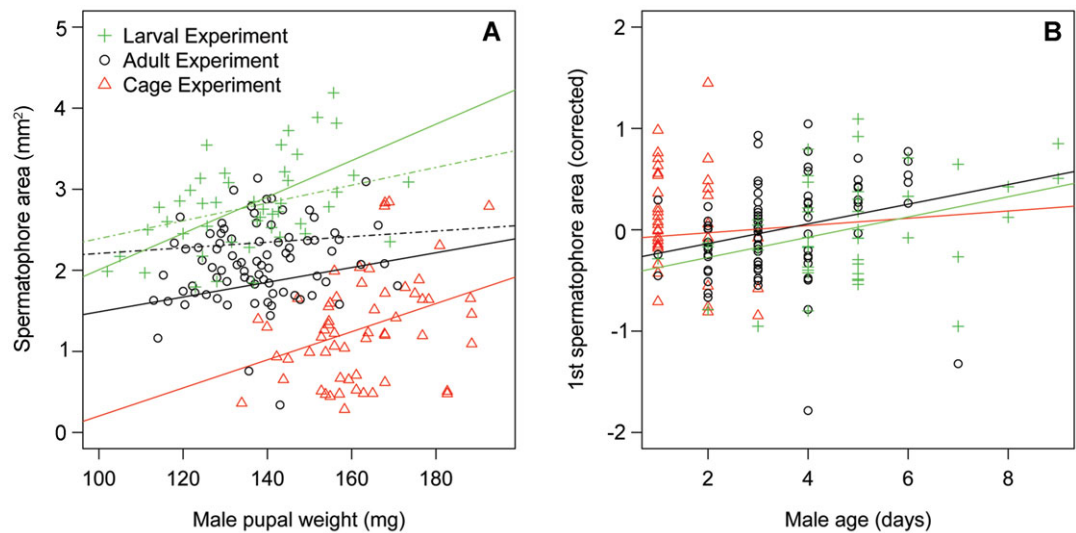


Fig. 1 The effect of (A) male pupal weight (mg) and (B) male age at first mating on the spermatophore surface area (in mm² and corrected for male pupal weight, respectively) in the 3 experiments of this study. The plain and dashed lines represent food-restricted and control treatments, respectively, for the larval and adult food-restriction experiments.

Table 1 Average values (\pm standard error) for male pupal weight (mg), spermatophore size (mm²), and mating period (min) from the different experimental sets.

Set 1: Copulation length and female reproductive output

	First mating	Second mating	Third mating
Spermatophore size (mm ²)	1.45 (\pm 0.1)	1.08 (\pm 0.2)	0.72 (\pm 0.2)
Mating period (min)	78.6 (\pm 8.8)	213.3 (\pm 51.1)	139.5 (\pm 34.7)
Male pupal weight (mg)	162.7 (\pm 2.3)	162.9 (\pm 3.2)	160.9 (\pm 4.3)
	Monandrous	Polyandrous	
Spermatophore size (mm ²)	1.32 (\pm 0.1)	1.27 (\pm 0.15)	
First spermatophore size (mm ²)	1.32 (\pm 0.1)	1.55 (\pm 0.23)	
First mating period (min)	102.6 (\pm 16)	118.5 (\pm 35)	

Set 2: Larval and adult food-restriction and male age at mating

	Control	Larval food-restricted
Spermatophore size (mm ²)	2.81 (\pm 0.09)	2.76 (\pm 0.08)
Male pupal weight (mg)	134.2 (\pm 6.1)	125.6 (\pm 5.9)
	Control	Adult food-restricted
Spermatophore size (mm ²)	2.38 (\pm 0.06)	1.83 (\pm 0.07)
Male pupal weight (mg)	139.3 (\pm 7.3)	133.1 (\pm 2.8)

Note: In Set 1, traits were compared between first, second, and third mating of the males, or between monandrous and polyandrous females. In Set 2, we compared spermatophore size and male pupal weight from controls and individuals that were food-restricted at larval or adult stage.

length of the first mating did not affect the size of the first spermatophore (Fig. 2). Virgin and nonvirgin females also received spermatophores of similar sizes (linear mixed model, $F_{1,9} = 2.45$, $P = 0.15$, data not shown). Finally,

although the number of clutches laid by a female increased with the size of the first spermatophore received by the female, the correlation was not significant for our dataset (linear model, $df = 1$, $F = 0.92$, $P = 0.35$, data not

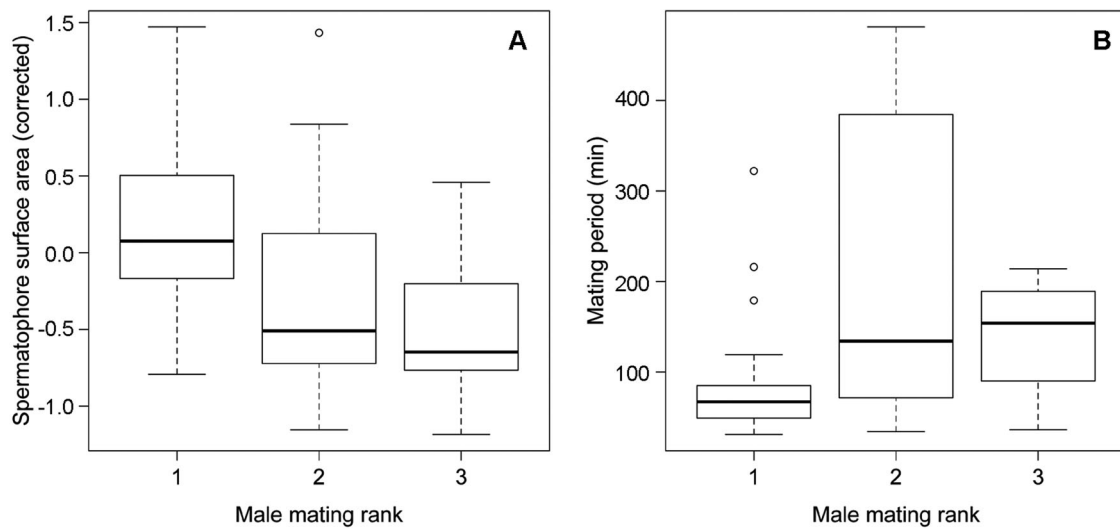


Fig. 2 Male mating rank effect on (A) spermatophore surface area (corrected for male pupal weight) and (B) copulation period (min).

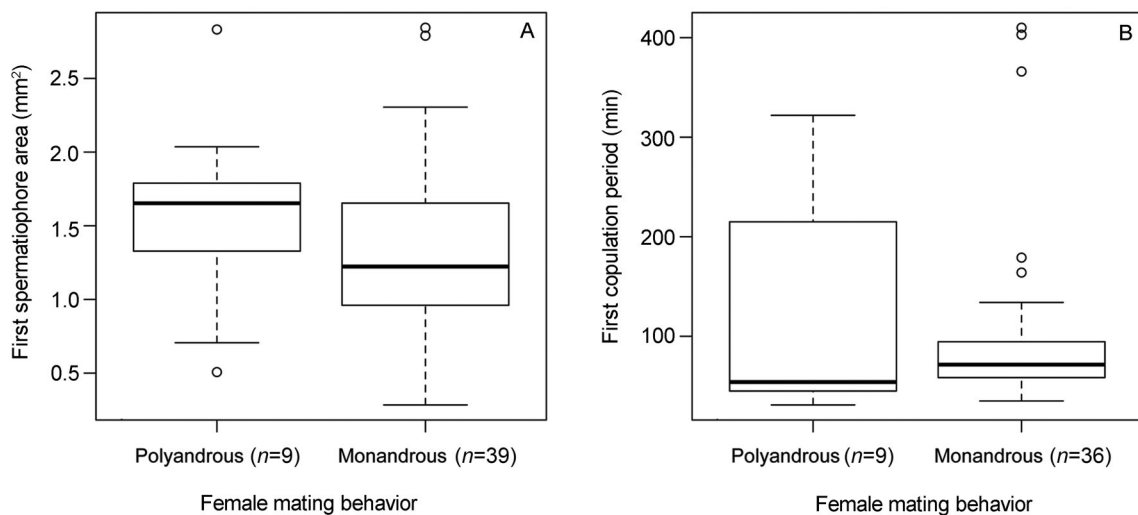


Fig. 3 Polyandrous versus monandrous females. (A) Surface area (mm²) of first spermatophore, and (B) copulation period (min) versus female remating behavior.

shown). There was no effect of the spermatophore size on the average egg hatching rate nor the hatch rate of the first clutch only (linear mixed model, $F_{1,4} = 0.51$, $P = 0.51$ and $F_{1,4} = 1.65$, $P = 0.27$, respectively, data not shown).

The spermatophores found in monandrous females were of similar size to the first spermatophore received by remated females (linear model, $df = 1$, $F = 1.19$, $P = 0.28$, Fig. 3A). The length of copulation involving a monandrous female was similar to the first copulation period of polyandrous females (Kruskal–Wallis test, $\chi^2 = 0.15$, $df = 1$, $P = 0.70$, Fig. 3B).

Set 2: Larval and adult food-restriction and male age at mating

We found that butterflies that were food-restricted during their final larval instar were not significantly lighter at pupal stage (linear mixed model, $F_{1,35} = 0.81$, $P = 0.38$, Fig. 4A). Males that were food-restricted at adult stage were also of similar pupal weight to the control males (linear mixed model, $F_{1,109} = 0.43$, $P = 0.51$, Fig. 4A). The interaction between life stage and food-restriction treatment was significantly affecting spermatophore size (linear mixed model, $F_{1,106} = 9$, $P = 3.4e-3$). Males that were food-restricted at

larval stage produced spermatophores of similar size to control males (Tukey's test, $P = 0.99$, Fig. 4B). In contrast, males that were food-restricted at the adult stage produced smaller spermatophores than control males (Tukey's test, $P < 0.001$, Fig. 4B).

Sperm image microscopy

As expected, we observed both fertile (eupyrene) and infertile (apyrene) sperm types in the dissected spermatophores of the Glanville fritillary butterfly (Fig. 5).

Discussion

We show that in the Glanville fritillary butterfly the size of the spermatophore is positively correlated with the age

of the male at his first mating. These results suggest that with reduced chance of multiple matings, due to increased senescence, males may invest more in their first mating to ensure maximal paternity from this potentially unique copulation (i.e., terminal investment hypothesis, with increased investment in current rather than future reproduction). The production of a large first spermatophore from older virgin males is also observed in *Bicyclus anynana* (Kehl *et al.*, 2015) and in *Pieris rapae* (Wedell & Cook, 1999), and results in a higher female reproductive success in *B. anynana* compared to the spermatophores of younger virgin males (Kehl *et al.*, 2015). Consequently, as both older nonmated males and young males produce large-sized spermatophores, developing the ability of distinguishing between young and old mates would not provide the correct clues on their mating status or on the

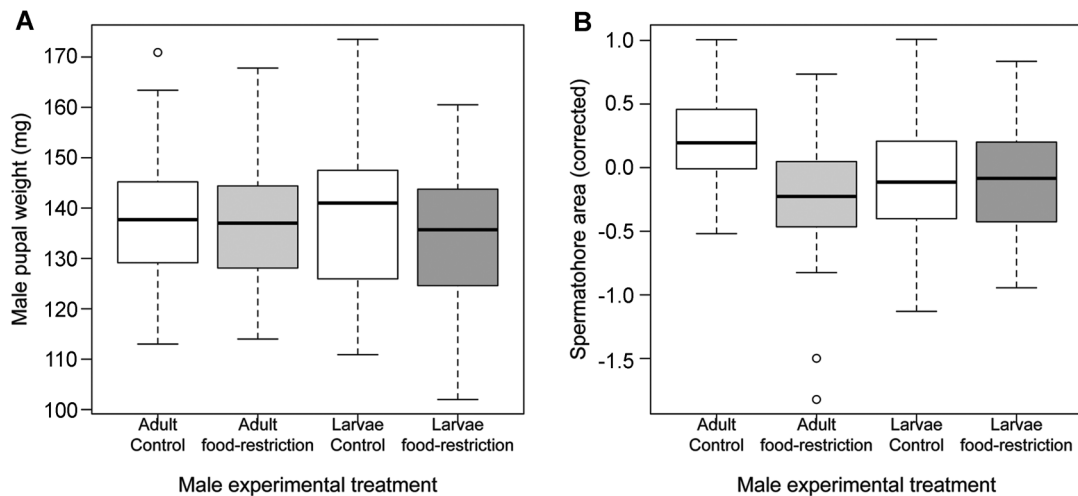


Fig. 4 The effect of larval and adult food-restrictions (white boxes for control, light gray and dark gray boxes for larval and adult food-restrictions, respectively) on (A) male pupal weight (mg) and (B) spermatophore surface area (corrected for male pupal weight).

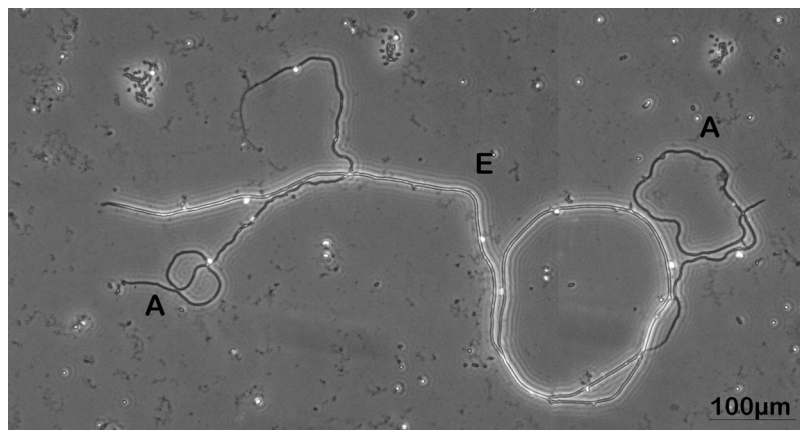


Fig. 5 Dark field phase imaging of the Glanville fritillary sperm cells. (E) Eupyrene sperm and (A) apyrene sperm (63× magnification).

size of the spermatophore to be received by the female. Hence, in general, selection should not act on the female preference toward males of a certain age category (old vs. young), but rather favor females with the ability of identifying the reproductive capacity or reproductive history of their mate. Female butterflies are known to be able to choose their mate based on various cues, often either visual or olfactory. In the Common grass yellow butterfly, *Eurema hecabe*, and in the Pipevine swallowtail, *Battus philenor*, females are attracted to males with bright iridescence spots on their wings, which, in the Pipevine swallowtail, act as indicators of males that provide larger spermatophores (Kemp, 2007; Rajyaguru *et al.*, 2013). In *B. anynana*, females make their choice based on the male's pheromones (Nieberding *et al.*, 2012) rather than on wing colors. In any case, female preference in regard to male reproductive capacity remains to be assessed in the Glanville fritillary butterfly.

Food-restriction during larval development (e.g., due to drought or disease) or at adult reproductive stage (e.g., due to asynchrony between adult emergence and nectar flowers availability) is likely to have a very different impact on the size and/or quality of the individuals. In the Glanville fritillary butterfly, males tend to produce a spermatophore of a size in accordance to their pupal size, which is often in accordance to the resources acquired at larval stage. In our larval food-restriction experiment, food deprived males were able to compensate for the 2 d of starvation in terms of their size as there was no difference in pupal weight between control food-restricted males. The larval food-restriction and the observed consecutive compensation in growth in these individuals did not seem to influence the reproductive investment of males. This is contrasting with previous results from Saastamoinen *et al.* (2013), showing a strong effect of larval food-limitation on the reproductive output of females in the Glanville fritillary butterfly after using the same 2-d larval food-restriction treatment as we did in our experiment. In this previous study, although food-restricted female larvae developed slower to reach a similar size than their nonfood-restricted counterparts, the compensating females had lower fecundity (smaller clutches laid) and shorter lifespan. This is suggestive of different resource-allocation strategies between male and female butterflies on their reproduction during developmental stages. Although females seem to invest the resources they acquired at larval stage mainly toward reproduction, it is not clear yet toward what primary purpose these same resources are invested in males.

In contrast, food-restriction experienced at the adult stage had greater impact on the size of the spermatophore produced than did the larval food-restriction treatment.

Our results hence suggest that the resources used for the quality, size and potentially also the production of additional spermatophores are mainly gathered during the adult stage. Such resources include amino acids, sugars or salts from flower nectar or damp mineral-rich soil (Lederhouse *et al.*, 1990). The quality and quantity of such natural butterfly food resources are affected by different abiotic and biotic factors in the wild, such as temperature and humidity, presence of competitors and pathogens. Sugar and amino acid-rich diets, provided at adult stage, were previously identified as essential to the production of large spermatophores in the swallowtail butterfly, *Papilio xuthus* (Watanabe & Hirota, 1999), and the Eastern tiger swallowtail, *P. glaucus* (Lederhouse *et al.*, 1990). In *P. glaucus*, adult males on amino acid-rich diet produced 7 times more larvae than control males (Lederhouse *et al.*, 1990). Similarly, in *B. anynana*, adult male butterflies fed on rotten fruits produce larger first spermatophores (Lewis & Wedell, 2007), and when fed of a sodium-rich solution show higher egg hatching success (Molleman *et al.*, 2004).

In contrast to other butterflies (Bissoondath & Wiklund, 1996; but see Jones *et al.*, 1986 for similar results in the Edith's checkerspot butterfly, *Euphydryas editha*; Wedell & Cook, 1998), an increased size of the spermatophore in the Glanville fritillary butterfly does not predict an increased paternity or higher male fertilization success. This is consistent with Duploup and Hanski (2015), who found no effect of spermatophore size on the fertility of males from the Åland Islands, and with the prediction that the spermatophore does not function as a nuptial gift in the Glanville fritillary butterfly. However, there are some indications, that the small spermatophore size may be an indicator of low quality males in the Glanville fritillary, as Duploup and Hanski (2015) showed on the island of Pikku-Tytarsaari (PT) in the Gulf of Finland. The PT individuals have accumulated a high genetic load through several generations of population isolation and inbreeding (Mattila *et al.*, 2012), leading to the production of small spermatophore by the PT males and consequent lower fertility in comparison to Åland males (Duploup & Hanski, 2015). Nonetheless, the spermatophore size decreases with female age and the number of clutches they lay in this species (Duploup & Hanski, 2015), suggesting that females digest the spermatophore and potentially use this valuable long-term resource to other means (e.g., longevity, dispersal, offspring survival to pupation).

For male butterflies, higher reproductive success does not only happen through directly enhancing their mate's reproductive output, but also through ensuring the paternity of a higher proportion of the offspring of the female

they have sired, compared to other male competitors with which the female may later remate with (Andersson *et al.*, 2000; Arnqvist & Rowe, 2005). In many Lepidoptera, male–male competition may be strong enough to induce the evolution of adaptive strategies. For example, in many species, female sexual receptivity is at least partially controlled by the male, which either uses the volume of the spermatophores, transfer nonfertile sperm-rich ejaculate, antiaphrodisiac hormones or mating-plugs to extend the refractory period of the female, and make them less susceptible to remate (Gilbert, 1976; Sugawara, 1979; Kaitala & Wiklund, 1994; Andersson *et al.*, 2000, 2004). There is no prior indication of mating-plug in the Glanville fritillary butterfly (Wahlberg, 1995). In contrast, previous studies showed that females previously mated with non-virgin males, tend to seek for additional male-derived resources that were lacking from the spermatophore of their nonvirgin mate, through remating (in *M. cinxia*: Duploux *et al.*, 2013; In *P. napi*: Kaitala and Wiklund, 1994; in *B. anynana*: Karl & Fisher, 2013). As the remains of the first spermatophores found in both monandrous and polyandrous females of the Glanville fritillary butterfly were of similar size, we suggest that the females originally received similar sized-spermatophores, and therefore that the female remating behavior in this species might be dictated by other quality factor(s) than the size of the spermatophore they received (e.g., the ratio of eupyrene vs. apyrene sperm in the ejaculate). This contrasts with results from Sugawara (1979) showing that in *P. rapae*, the mechanical stretch of the bursa copulatrix due to the transfer of a spermatophore correlates with the female's remating behavior. We show that the copulation length does not correlate with the size of the spermatophore transferred to the female. It is possible that in the Glanville fritillary butterfly, some males may use their own bodies as a mating-plug (i.e., in-copula mate-guarding strategy), thus to avoid sperm competition and ensure fertilization of the female eggs (Svärd & Wiklund, 1988). Long copulation time with a fast sperm transfer phase have been observed in other insects (Parker, 1970; Svärd & Wiklund, 1988), suggesting that males could transfer their spermatophore in the first half of the copulation period and use the rest of the time to guard the female from remating immediately after the transfer of the spermatophore from the male. Such mate-guarding behavior remains to be investigated in the Glanville fritillary butterfly. Our results however indicate that the length of the first copulation does not affect the female remating behavior but rather increases with the male mating status. Long copulations might therefore only be the characteristic of tired males, and not an adaptation toward the reduction of the risk of sperm competition in the female. Finally, it is possible that females digest

the spermatophores they received at different rates, thus suggesting that originally the females might receive spermatophores of different sizes or quality. Although we have no way to calculate the amount of the spermatophore digested by the female, such difference, if it exists, could also explain variations in both mating period and female remating behaviors.

Conclusion

Our study provides new insight into various male butterfly characteristics that may affect the quality of the spermatophore and impede both male and female reproductive success. We show that the spermatophore size of the Glanville fritillary male butterflies increases with male weight at pupal stage and male age at first mating, but decreases with male food-restriction at adult stage and male frequency of mating. These results suggest that males are resource restricted while producing the spermatophore and should allocate it wisely in populations where females are rare or where male-male competition is high (Svärd & Wiklund, 1988; Watanabe *et al.*, 1997; Charlat *et al.*, 2007). We also show that in this species, spermatophore size is not correlated with the length of the copulation nor with the reproductive output of the female. The previously reported observation that a female receiving a smaller spermatophore from nonvirgin males tends to seek for a second mate (Sarhan & Kokko, 2007; Duploux *et al.*, 2013) however suggests that under certain circumstances, females may seek for additional male-derived reproductive material. In contrast with other species (Boggs & Gilbert, 1979; Bonoan *et al.*, 2015), it is yet unclear what part(s) of the spermatophore is used by the Glanville fritillary female butterflies and to what final purpose.

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Disclosure

There are no conflicts of interest concerning this article.

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